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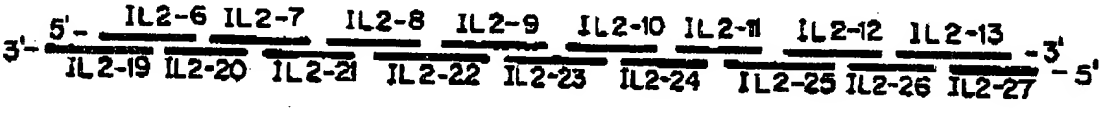
(54) Title: INTERLEUKIN-2 PRODUCTION USING CLONED GENES FOR INTERLEUKIN-2 AND YEAST ALPHA-FACTOR



(57) Abstract

Methods and compositions for efficient production of human interleukin-2. A synthetic interleukin-2 gene is joined to yeast alpha-factor secretory leader and processing signals to provide for expression and secretion of mature gene product in yeast. Enhanced yields of the product may be recovered from the nutrient medium.

INTERLEUKIN-2 PRODUCTION USING CLONED GENES FOR INTERLEUKIN-2 AND YEAST ALPHA-FACTOR

Background of the Invention

1. Field of the Invention

Lymphokines are naturally-occurring polypeptides, produced by normal lymphocytes, which mediate the host's immune response to antigenic challenge. A particular lymphokine, interleukin-2, appears to promote the host's immune response and has potential value in the treatment of tumors, immuno-deficiency diseases and several other clinical conditions, and as an adjuvant for vaccine administration. Interleukin-2 appears to act as a potent mitogen for T lymphocytes. At present, only limited quantities of interleukin-2 are obtained by separation from human serum or from certain human cell tissue culture media. It is therefore of great scientific and clinical importance to be able to produce sufficiently large quantities of interleukin-2. Economic, efficient methods for producing a product having the properties of mature human interleukin-2 have therefore become significant goals.

2. Description of the Prior Art

Kurjan and Herskowitz, Cell (1982) 30:933-934 describe a putative α-factor precursor containing four tandem copies of mature α-factor, describing the sequence and postulating a processing mechanism. Kurjan and Herskowitz, Abstracts of Papers presented at the 1981 Cold Springs Harbor Meeting on The Molecular Biology of Yeast, p. 242, in an Abstract entitled "A Putative Alpha-Factor Precursor Containing Four Tandem

Repeats of Mature Alpha-Factor," describe the sequence encoding for the α-factor and spacers between two of such sequences.

Taniguchi et al., Nature (1983) 302:305-310, report the cloning of a human interleukin-2 cDNA and its expression in monkey cells in tissue culture, describe the oligonucleotide sequence and give an inferred amino acid sequence for both a putative precursor and presumed mature form. Robb et al., (1983) Proc. Natl. Acad. Sci USA 80:5990-5994 describe a partial amino acid sequence from the N-terminal region of mature human interleukin-2 isolated from JURKAT cell tissue culture medium.

Summary of the Invention

Novel methods and DNA constructs are provided for the production of polypeptides having biological activity analogous to interleukin-2 (IL2). Enhanced efficiency in production of the polypeptide is achieved, in part, by the complete synthesis of the structural gene employing codons preferentially utilized by yeast, the intended host. Desirably, at least about 50%, usually, at least about 60% of the codons are modified such that most of the codons of the structural gene are those preferentially utilized by yeast. The construct includes a replication system for yeast and the structural gene joined in reading frame to secretory and processing signals recognized by yeast. The yeast host transformant provides for efficient and economic production of a product useful as interleukin-2.

Brief Description of the Drawings

Fig. 1 illustrat s the order of assembly of synthetic ssDNA segments used in preparing the 5'-half of the synthetic IL2 gene.

Fig. 2 illustrates the scheme utilized to clone the synthetic DNA fragment of Fig. 1.

Fig. 3 illustrates the order of assembly of ssDNA segments used in preparing the 3'-half of the synthetic IL2 gene.

Fig. 4 illustrates the scheme utilized to clone the synthetic gene fragment of Fig. 3.

Fig. 5 illustrates the scheme utilized to join the two halves of the synthetic IL2 gene.

Description of the Specific Embodiments

DNA constructs capable of expressing mammalian, particularly human interleukin-2 (IL2) in a eukaryotic microorganism host are provided. (Unless otherwise indicated when referring to interleukin-2 (IL2) polypeptide(s), it is intended to include not only the naturally occurring mammalian factors, but also fragments or analogs thereof exhibiting analogous biological activity.) These DNA fragments can be incorporated into vectors, and the resulting plasmids used to transform susceptible hosts. Transformation of a susceptible host with such recombinant plasmids results in expression of the IL2 gene and production of a mature polypeptide product having the physiological and immunological activity of IL2. That is, it acts in the same manner as IL2 isolated from either a rat or a human host in recognized bioassays.

Extrachromosomal constructs are provided having as essential elements for expression of mature polypeptides, a replication system recognized by yeast, a synthetic structural gene having a plurality of

codons preferentially utilized by yeast, the structural gene being in reading frame with efficient secretory leader and processing signals to provide for the efficient secretion and processing of the polypeptide in a yeast host, and production of a product in high yield which has biological, particularly immunological and physiological activity commensurate with human interleukin-2. The construct provides for the initial formation of "pre"-IL2.

By "pre"-IL2, it is meant that the DNA sequence encoding the mature polypeptide is joined to and in reading frame with a leader sequence including processing signals efficiently recognized by the yeast host. Thus, "pre" denotes the inclusion of secretory leader and processing signals sequences (on the precursor polypeptide) recognized by yeast and does not refer to any processing signals associated with the natural human IL2 gene.

The IL2 structural gene of this invention is synthetic DNA, which is prepared using codons preferred by yeast as evidenced by the codon frequency in structural genes encoding for yeast glycolytic enzymes. The secretory leader and processing signals are conveniently derived from naturally-occurring DNA sequences in yeast, which sequences provide for the secretion and processing of polypeptide(s). Such polypeptides which are naturally secreted by yeast include α-factor, a-factor, acid phosphatase, and the like. The remaining sequences in the construct, including the replication system, promoter, and terminator, are well known and amply described in the literature.

In preparing the DNA constructs of the present invention, it is necessary to bring the individual DNA sequences embodying the structural gene, the secretory leader and processing signals, replication system, promoter, and terminator together

in a predetermin d order to assure that th y are able to properly function in the r sulting plasmid. Since the various DNA sequences which are joined to form the DNA construct of the present invention will be derived from diverse sources, in some instances it will be convenient or necessary to join the sequences by means of connecting or adaptor molecules, which in the subject invention are incorporated into the synthetic gene.

In developing the subject invention, advantage is taken of a pre-existing vector, which includes a multicopy number yeast replication system, a bacterial replication system, appropriate markers for selection, as well as a promoter, transcriptional terminator and modified leader sequence associated with the secretion of α-factor. See copending application Serial No. 522,909, filed August 12, 1983.

The structural gene encoding for IL2 is prepared by first preparing a series of single stranded fragments ranging in number of from about 10 to 40, conveniently 14 to 34 bases, which provide for overlapping of other fragments and overhangs, so that upon bringing together the fragments under ligating conditions, dsDNA is produced having appropriate cohesive ends. In view of the large size of the IL2 structural gene and to provide for future flexibility in potential manipulations, a 5'- and 3'-fragment were prepared and then combined to provide for a structural gene encoding the entire amino acid sequence of IL2 and providing for appropriate termini for linking to restriction sites in the vector, which provide for the structural gene being in frame with the secretory leader sequence.

Each of the fragments encoding for the gene - 5'-fragment and 3'-fragment with flanking regions as appropriate - may be cloned and amplified in an appropriate vector to expand the amount of the fragment

and to ensure its integrity. The 5'-fragment is inserted downstream from transcriptional regulatory signals for transcription initiati n and a secretory leader sequence which includes a convenient restriction site at or about the processing signal.

By employing a synthetic fragment, the termini of the fragment can be tailored to fulfill the requirements necessary for subsequent processing steps. For the 5'-fragment, its 5'-terminus is designed to join to the secretory leader sequence and processing signal in reading frame and to replace any nucleotides which have been lost due to restriction or other processing of the nucleotide sequence encoding for the secretory leader and processing signal. Thus, in restricting the vector, a restriction site can be chosen which is internal to the coding region for the secretory leader and processing signal.

In addition, it may be useful to extend the 5'-fragment in the 3'-direction beyond the site where the 5'-fragment and 3'-fragment are to be linked. In this way, a convenient 3'-terminus of the 5'-fragment is present for joining to the vector 5'-terminus. The extension is then removed by restriction to provide a 3'-terminus of the 5'-fragment which is complementary to the 5'-terminus of the 3'-fragment.

Parallel manipulations may be employed with the 3'-fragment to provide for appropriate restriction sites and termini for joining to a vector and to the 5'-fragment, as well as supplying nucleotides in the 3'-non-coding region associated with termination of transcription and translation.

After cloning the synthetic 5'-fragment in the vector containing the secretory leader sequence, a new extended 5'-fragment is excised. The new 5'-fragment has its 5'-end beginning with the transcriptional regulatory signals controlling the transcription of the secretory leader sequence and

processing signal, followed by the synthetic 5'-fragment in reading frame with the secret ry leader sequence. Thus, this n w fragment now includ s the promoter and other associated transcriptional regulatory sequences, such as the TATA box and capping sequence, as well as any other sequences involved in the efficient transcription of α-factor. By employment of appropriate restriction enzymes, the fragment is obtained which has all the necessary transcriptional regulatory functions, the secretory leader and processing signal, the 5'-end of the IL2 structural gene and a cohesive end or blunt end for ligating to the 5'-terminus of the 3'-fragment.

The 3'-half of the IL2 is inserted into an appropriate vector for cloning. The resulting plasmid has a unique restriction site at the 5'-end of the 3'-fragment resulting in cohesive termini when cut, and appropriate transcriptional termination sequences downstream, such as a terminator and polyadenylation signal. The fragment to be inserted conveniently has the same cohesive termini. In order to prevent circularization of the plasmid without insertion of the 5'-end of the IL2, the plasmid is treated with phosphatase. The fragment containing the transcriptional regulatory signals and 5'-end of the IL2 structural gene is then inserted into the phosphatase-treated plasmid for bacterial transformation and subsequently excised and ligated to provide for a plasmid capable of transforming a yeast host efficiently, being multicopy, and providing for the efficient secretion of the polypeptide encoded by the IL2 gene.

While the homologous promoter associated with the secretory leader sequence may be used, it may also be replaced with other promoters or may be used in tandem with other promoters.

A wide variety of promoters are available or can be btained from y ast genes. Promoters of

particular interest include acid phosphatas and those promoters involved with enzymes in the glycolytic pathway, such as promoters for alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, triose phosphate isomerase, phosphoglucoisomerase, phosphofructokinase, etc. By employing these promoters with regulatory sequences, such as enhancers, operators, etc., and using a host having an intact regulatory system, one can regulate the expression of the "pre"-IL2, and various small organic molecules, e.g., glucose, may be employed for the regulation of production of the desired polypeptide.

One may also employ temperature controlled sytems, e.g., temperature-sensitive regulatory mutants which allow for modulation of transcription by varying the temperature. Thus, by growing the cells at either the non-permissive or permissive temperature, as appropriate, one can grow the cells to high density, before changing the temperature in order to provide for expression of the "pre"-polypeptide for IL2.

Other capabilities may also be introduced into the construct. For example, some genes provide for amplification, where upon stress to the host, not only is the gene which responds to the stress amplified, but also flanking regions. By placing such a gene upstream from the promoter, coding region and the other regulatory signals providing transcriptional control of the "pre"-polypeptide, and stressing the yeast host, plasmids may be obtained which have a plurality of repeating sequences, which sequences include the "pre"-polypeptide gene with its regulatory sequences. Illustrative genes include metallothioneins and dihydrofolate reductase.

The construct may include, in addition to the secretory leader and processing signal sequence, other DNA homologous to the host genome. If it is desired that there be integration of the IL2 gene into the

chromosome(s), integration can be enhanced by pr viding f r sequences flanking th IL2 gene construct which are homologous to host chromosomal DNA.

The replication system which is employed will be recognized by the yeast host. Therefore, it is desirable that the replication system be native to the yeast host. A number of yeast vectors are reported by Botstein et al., Gene (1979) 8:17-24. Of particular interest are the YEp plasmids, which contain the 2μm plasmid replication system. These plasmids are stably maintained at multiple copy number. Alternatively, or in addition, one may use a combination of ARS1 and CEN4, to provide for stable maintenance.

The plasmids may be introduced into the yeast host by any convenient means, employing yeast host cells or spheroplasts and using DNA for transformation, or liposomes, or calcium precipitated DNA or other conventional technique. The modified hosts may be selected in accordance with the genetic markers which are usually provided in a vector used to construct the expression plasmid. An auxotrophic host may be employed, where the plasmid has a gene which complements the host and provides prototrophy. Alternatively, resistance to an appropriate biocide, e.g., antibiotic, heavy metal, toxin, or the like, may be included as a marker in the plasmid. Selection may then be achieved by employing a nutrient medium which stresses the parent cells, so as to select for the cells containing the plasmid. The plasmid containing cells may then be grown in an appropriate nutrient medium, and the desired secreted polypeptide isolated in accordance with conventional techniques. The polypeptide may be purified by chromatography, filtration, extraction, etc. Since the polypeptide will be present in mature form in the nutrient medium, one can cycle the nutrient medium, continuously removing the desired polypeptide.

The following examples are offered by way of example and not by way of limitation.

EXPERIMENTAL

A nucleotide sequence for interleukin-2 comprising preferentially utilized yeast codons was devised. The sequence included a portion of a modified α-factor secretory leader and processing signal at its 5'-end. The three glu-ala pairs were deleted and a base pair was changed internal to the secretory leader sequence to promote a KpnI site and change the codon from ser to gln. The sequence with the coding strand shown 5' to 3', was as follows:

```
                    Processing Site
      GlnLeuAspLysArgAlaProThrSerSerSerThrLysLysThrGlnLeuGlnLeu
   5'-AGCTGGATAAAAGAGCTCCAACCTCTTCCTCTACCAAGAAGACCCAGCTGCAATTG
3'-CATGTCGACCTATTTTCTCGAGGTTGGAGAAGGAGATGGTTCTTCTGGGTCGACGTTAAC
   (KpnI)

   GluHisLeuLeuLeuAspLeuGlnMetIleLeuAsnGlyIleAsnAsnTyrLysAsnPro
   GAACACTTGTTGTTGGACTTGCAAATGATCTTGAACGGTATCAACAACTACAAGAACCCA
   CTTGTGAACAACAACCTGAACGTTTACTAGAACTTGCCATAGTTGTTGATGTTCTTGGGT

   LysLeuThrArgMetLeuThrPheLysPheTyrMetProLysLysAlaThrGluLeuLys
   AAGTTGACCAGAATGTTGACCTTCAAGTTCTACATGCCAAAGAAGGCTACCGAATTGAAG
   TTCAACTGGTCTTACAACTGGAAGTTCAAGATGTACGGTTTCTTCCGATGGCTTAACTTC

   HisLeuGlnCysLeuGluGluGluLeuLysProLeuGluGluValLeuAsnLeuAlaGln
   CACCTGCAGTGTCTAGAGGAAGAGTTGAAGCCATTGGAAGAAGTCCTGAACTTGGCTCAA
   GTGGACGTCACAGATCTCCTTCTCAACTTCGGTAACCTTCTTCAGGACTTGAACCGAGTT
            XbaI

   SerLysAsnPheHisLeuArgProArgAspLeuIleSerAsnIleAsnValIleValLeu
   TCTAAGAACTTCCACTTGAGACCAAGAGACTTGATCTCTAACATCAACGTTATCGTTTTG
   AGATTCTTGAAGGTGAACTCTGGTTCTCTGAACTAGAGATTGTAGTTGCAATAGCAAAAC

   GluLeuLysGlySerGluThrThrPheMetCysGluTyrAlaAspGluThrAlaThrIle
   GAATTGAAGGGTTCTGAAACCACCTTCATGTGTGAATACGCTGACGAAACCGCTACCATC
   CTTAACTTCCCAAGACTTTGGTGGAAGTACACACTTATGCGACTGCTTTGGCGATGGTAG

   ValGluPheLeuAsnArgTrpIleThrPheCysGlnSerIleIleSerThrLeuThrOP
   GTTGAATTCTTGAACAGATGGATCACCTTCTGTCAATCTATCATCTCTACCTTGACCTGA
   CAACTTAAGAACTTGTCTACCTAGTGGAAGACAGTTAGATAGTAGAGATGGAACTGGACT
```

AM
TAGGCGTCG-3'
ATCCGCAGCAGCT-5'
SalI

The sequence is provided with a KpnI cohesive end at the 5'-end and a SalI cohesive end at the 3'-end. The coding sequence for the mature polypeptide begins after the processing site.

A synthetic DNA fragment for interleukin-2 having the above sequence was prepared by synthesizing and cloning two halves of the fragment separately. Each half fragment was prepared by synthesizing overlapping ssDNA segments using the phosphoramidite method, as described by Beaucage and Caruthers (1981) Tetrahedron Lett. 22:1859-1862. The ssDNA segments were as follows:

Designation	Sequence (5' to 3')
Linker	AGCTGGATAAAAGA
IL2-1	GCTCCAACCTCTTCCTCTACCAAGAAGACCCAG
IL2-2	CTGCAATTGGAACACTTGTTGTTGGACTT
IL2-3	GCAAATGATCTTGAACGGTATCAACAACTACA
IL2-4	AGAACCCAAAGTTGACCAGAATGTTGACCTTC
IL2-5	AAGTTCTACATGCCAAAGAAGGCTACCGAATT
IL2-6	GAAGCACCTGCAGTGTCTAGAGGAAGAGTTG
IL2-7	AAGCCATTGGAAGAAGTCCTGAACTTGGCTCAAT
IL2-8	CTAAGAACTTCCACTTGAGACCAAGAGACTT
IL2-9	GATCTCTAACATCAACGTTATCGTTTTGGAAT
IL2-10	TGAAGGGTTCTGAAACCACCTTGATGTGTGAA
IL2-11	TACGCTGACGAAACCGCTACCATCGTTGAAT
IL2-12	TCTTGAACAGATGGATCACCTTCTGTCAATC
IL2-13	TATCATCTCTACCTTGACCTGATAGGCGTCG
IL2-14	GAAGAGGTTGGAGCTCTTTTATCCAGCTGTAC
IL2-15	GTTCCAATTGCAGCTGGGTCTTCTTGGTAGAG
IL2-16	GTTCAAGATCATTTGCAAGTCCAACAACAAGT
IL2-17	GTCAACTTTGGGTTCTTGTAGTTGTTGATACC
IL2-18	TTGGCATGTAGAACTTGAAGGTCAACATTCTG
IL2-19	ACACTGCAGGTGCTTCAATTCGGTAGCCTTCT
IL2-20	TTCTTCCAATGGCTTCAACTCTTCCTCTAG

IL2-20'	TCGACAACTCTTCCTCTAG
IL2-21	AGTGGAAGTTCTTAGATTGAGCCAAGTTCAGGAC
IL2-22	GTTGATGTTAGAGATCAAGTCTCTTGGTCTCA
IL2-23	GTTTCAGAACCCTTCAATTCCAAAACGATAAC
IL2-24	GGTTTCGTCAGCGTATTCACACATGAAGGTG
IL2-25	TCCATCTGTTCAAGAATTCAACGATGGTAGC
IL2-26	AAGGTAGAGATGATAGATTGACAGAAGGTGA
IL2-27	TCGACGACGCCTATCAGGTC

The 5'-half of the sequence was assembled as illustrated in Fig. 1. Fifty pmoles of each ssDNA segment (except Linker and IL2-20') were 5'-phosphorylated with T4 polynucleotide kinase. The segments were then annealed in a single step by combining and cooling from 95°C to 25°C over 1.5 hours. Ligation was performed in a reaction volume of 30µl containing 1mM ATP, 10mM DTT, 100mM tris-HCl, pH 7.8, 10mM $MgCl_2$, 1µg/ml spermidine and T4 ligase. The resulting double stranded fragment was purified on a 7% native polyacrylamide electrophoresis gel. The dsDNA fragment included a KpnI cohesive end at the 5'-end and a SalI cohesive end at the 3'-end.

After assembly, the 5'-half of the sequence was inserted into paEGF-24 downstream and in frame with the modified α-factor secretory leader and processing signal. The plasmid paEGF-24 is described in application serial no. 522,909, filed August 12, 1983, which pertinent portion is incorporated by reference and reproduced in pertinent part as follows.

A synthetic sequence for human epidermal growth factor (EGF) based on the amino acid sequence of EGF reported by H. Gregory and B. M. Preston Int. J. Peptide Protein Res. 9, 107-118 (1977) was prepared. The sequence was inserted into the EcoRI site of pBR328 to produce a plasmid p328EGF-1 and cloned.

Approximately 30µg of p328EGF-1 was digested with EcoRI and approximately 1µg of the expected 190

Herskowitz, Abstracts 1981 Cold Spring Harbor meeting on the Molecular Biology of Yeasts, page 242).

The resulting mixture was used to transform E. coli HB101 cells and plasmid pAB201 obtained. Plasmid pAB201 (5µg) was digested to completion with the enzyme EcoRI and the resulting fragments were:

a) filled in with DNA polymerase I Klenow fragment;

b) ligated to an excess of BamHI linkers; and

c) digested with BamHI. The 1.75 kbp EcoRI fragment was isolated by preparative gel electrophoresis and approximately 100ng of the fragment was ligated to 100ng of pC1/1, which had been previously digested to completion with the restriction enzyme BamHI and treated with alkaline phosphatase.

Plasmid pC1/1 is a derivative of pJDB219, Beggs, Nature (1978) 275:104, in which the region corresponding to bacterial plasmid pMB9 in pJDB219 has been replaced by pBR322 in pC1/1. This mixture was used to transform E. coli HB101 cells. Transformants were selected by ampicillin resistance and their plasmids analyzed by restriction endonucleases. DNA from one selected clone (pYEGF-8) was prepared and used to transform yeast AB103 cells. Transformants were selected by their leu^+ phenotype.

The above described construction was modified using different sequences for joining structural genes to the α-factor secretory leader sequence and/or site specific mutagenesis, thus providing for different processing signals. In the following table, a. through e. show the sequence of the fusions at the N-terminal region of the structural gene hEGF, as exemplary, which sequences differ among the several constructions.

```
 80                  85                      90          /           5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Glu Ala Asn Ser Asp Ser Glu
                                                 Linker-1
GGG CTA TCT TTG GAT.AAA AGA GAG GCT GAA GCT GAA GCT AAC TCC GAC TCC GAA              (pYαEGF-21)
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA CTT CGA TTG AGG CTG AGG CTT
 80                  85                      90                      /           5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
                                                Linker-2
GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA  (pYαEGF-22)
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80                  85      /           5
Gly Val Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu

GGC CTA TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA    (pYαEGF-23)
CCC CAT AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80                  85      /           5
Gly Val Pro Leu Asp Lys Arg Asn Ser Asp Ser Glu
    KpnI
GGG CTA CCT TTG GAT AAA AGA AAC TCC GAC TCC GAA    (pYαEGF-24)
CCC CAT GGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 80                  85      /           5
Gly Val Gln Leu Asp Lys Arg Asn Ser Asp Ser Glu
        PvuII   Linker-3
GGG GTA CAG CTG GAT AAA AGA AAC TCC GAC TCC GAA    (pYαEGF-25)
CCC CAT GTC GAC CTA TTT TCT TTG AGG CTG AGG CTT
 50          53
Trp Trp Glu Leu Arg
                    Linker-4
TGG TGG GAA TTG AGA TGA TAA GTC GAC CGA TG
ACC ACC CTT AAC TCT ACT ATT CAG CTG GCT AC
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f. shows the sequences at the C-terminal region of hEGF, which is the same for all constructions. Synthetic oligonucl otide linkers used in these constructions are boxed.

These fusions were made as follows. Construction (a) was made as described above. Construction (b) was made in a similar way except that linker 2 was used instead of linker 1. Linker 2 modifies the α-factor processing signal by inserting an additional processing site (ser-leu-asp-lys-arg) immediately preceding the hEGF gene. The resulting yeast plasmid is named pYαEGF-22. Construction (c), in which the dipeptidyl aminopeptidase maturation site (glu-ala) has been removed, was obtained by in vitro mutagenesis of construction (a). A PstI-SalI fragment containing the α-factor leader-hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A synthetic 31-mer of sequence

5'-TCTTTGGATAAAAGAAACTCCGACTCCCG-3'

was synthesized and 70 picomoles were used as a primer for the synthesis of the second strand from 1 picomole of the above template by the Klenow fragment of DNA polymerase. After fill-in and ligation at 14° for 18 hrs., the mixture was treated with S_1 nuclease (5 units for 15 minutes) and used to transfect E. coli JM101 cells. Bacteriophage containing DNA sequences in which the region coding for (glu-ala) was removed were located by filter plaque hybridization using the ^{32}P-labelled primer as probe. RF DNA from positive plaques was isolated, digested with PstI and SalI and the resulting fragment inserted in pABl14 which had been previously digested to completion with SalI and partially with PstI and treated with alkaline phosphatase.

The plasmid pABl14 was derived as follows: plasmid pABl12 was digested to completion with HindIII and then religated at low (4µg/ml) DNA concentration

and plasmid pAB113 was obtained in which three 63bp HindIII fragments have been del ted from the α-f ct r structural gene, leaving only a single copy of mature α-factor coding region. A BamHI site was added to plasmid pAB11 by cleavage with EcoRI, filling in of the overhanging ends by the Klenow fragment of DNA polymerase, ligation of BamHI linkers, cleavage with BamHI and religation to obtain pAB12. Plasmid pAB113 was digested with EcoRI, the overhanging ends filled in, and ligated to BamHI linkers. After digestion with BamHI the 1500bp fragment was gel-purified and ligated to pAB12 which had been digested with BamHI and treated with alkaline phosphatase. Plasmid pAB114, which contains a 1500bp BamHI fragment carrying the α-factor gene, was obtained. The resulting plasmid (pAB114 containing the above described construct) is then digested with BamHI and ligated into plasmid pC1/1.

The resulting yeast plasmid is named pYαEGF-23. Construction (d), in which a new KpnI site was generated, was made as described for construction (c) except that the 36-mer oligonucleotide primer of sequence 5'-GGGTACCTTTGGATAAAAGAAACTCCGACTCCGAAT-3' was used. The resulting yeast plasmid is named pYαEGF-24. Construction (e) was derived by digestion of the plasmid containing construction (d) with KpnI and SalI instead of linker 1 and 2. The resulting yeast plasmid is named pYαEGF25.

The 5'-half of the IL2 sequence was inserted according to the scheme illustrated in Fig. 2. Plasmid pαEGF-24 was restricted with a mixture of restriction endonucleases KpnI and SalI to remove a KpnI/SalI fragment. The 5'-synthetic fragment of IL2 was inserted into the resulting cut vector to produce plasmid pαIL2-5' which was then cloned in E. coli HB101.

The 3'-half of the sequence was assembled as illustrated in Fig. 3. Fifty pmoles of each ssDNA

segment (except IL2-6 and IL2-27) were 5'-phosphorylated with T4 polynucleotide kinase. The segments were then annealed in a single step by combining and cooling from 95°C to 25°C over 1.5 hours. Ligation was performed in a reaction volume of 30µl containing 1mM ATP, 10mM DTT, 100mM tris-HCl, pH 7.8, 10mM $MgCl_2$, 1µg/ml spermidine and T4 ligase. The resulting double stranded fragment was purified on a 7% native polyacrylamide electrophoresis gel. The dsDNA fragment included a XbaI cohesive end at the 5'-end and a SalI cohesive end at the 3'-end.

After assembly, the 3'-half of the sequence was inserted in XbaI/SalI digested pAB114. Plasmid pAB114 is described in application serial no. 522,909 which has in part been reproduced above.

The 3'-half of the sequence was cloned according to the scheme illustrated in Fig. 4. Plasmid pAB114 was restricted with a mixture of restriction endonucleases XbaI and SalI to remove a XbaI/SalI fragment. The 3'-synthetic fragment was inserted into the resulting cut vector to produce pαIL2-3' which was then cloned in E. coli RB101.

After amplification, the two halves of the synthetic IL2 sequence in frame with yeast secretory and processing signals, as well as joined at the 5'-end to yeast transcriptional regulatory signals, were joined together in the pαIL2-3' plasmid according to the scheme illustrated in Fig. 5. Plasmid pαIL2-5' was restricted with restriction endonuclease XbaI to generate an XbaI/XbaI fragment carrying the α-factor transcriptional regulatory sequences and modified secretory leader and processing signals derived from plasmid pαEGF-24 and the 5'-half of the IL2 sequence. The 3'-proximal XbaI site in the 5'-segment is located interior of the synthetic sequence so that a 15 bp segment at the 3'-end is removed. Plasmid pαIL2-3' was

bioassay for human interleukin-2. A semi-quantative assay was employed by visually estimating cell survival and growth empl ying a microscop and comparing test results with standards employing known amounts of IL2.

Serial 2-fold dilutions of the yeast medium dialysate above were then prepared in mammalian cell growth medium (RPMI-1640 with supplements, as above) and 100 µl of each diluted sample added to individual wells containing HT-2 cells.

Reference standards known to contain interleukin-2 (concanavalin A-free, conditioned rat splenocyte medium) were either obtained commercially (Monoclone®, Collaborative Research, Inc.) or prepared by stimulation of rat spleen cell cultures (in RPMI-1640 medium with supplements, as above) with concanavalin A (1 µg/10^6 cells) for 48 hours at 37°C in 7% CO_2/air, followed by recovery of the medium, absorption with Sephadex® G-25 to remove concanavalin A and filter sterilization. A 2-fold dilution series of each reference standard was prepared as described for the yeast medium dialysate.

The HT-2 cell microplate cultures were incubated at 37°C in 7% CO_2/air for 48 hours, scored for viability and/or growth and the approximate interleukin-2 content of the yeast preparation determined by reference to the standards. This comparison indicated activities equivalent to or greater than the commercial material, i.e., estimated to be in the range of 20-100 ng/ml, probably about 50 ng/ml.

In accordance with the subject invention, novel DNA constructs are provided which may be inserted into vectors to provide for expression of "pre"-interleukin-2 and intracellular processing and secretion of the mature polypeptide in good yield to promote a polypeptide product having high IL2 biological activity in a recognized bioassay based on

murine cellular growth. Thus, it is possible to obtain a p lypeptide having the physiological activity of the naturally-occurring human interleukin-2. By providing for secretion, greatly enhanced yields can be obtained and subsequent isolation and purification of the polypeptides simplified.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for producing a biologically active polypeptide having th biological activity of human IL2 in good yield, said method comprising:

growing yeast transformants having a multicopy extrachromosomal element including a DNA construct which comprises in the direction of transcription, yeast recognized transcriptional regulatory sequences, and secretory leader and processing signals, in frame with said secretory leader and processing signals a synthetic gene encoding at least substantially for the polypeptide sequence of human IL2, stop codon(s) and a terminator, whereby a mature polypeptide having at least substantially the same amino acid sequence of human IL2 is secreted; and

isolating said mature polypeptide from the medium having the biological activity of human IL2.

2. A method according to Claim 1, wherein the coding strand of said synthesized sequence has the following nucleotide sequence:

GCTCCAACCTCTTCCTCTACCAAGAAGACCCAGCTGCAATTG

GAACACTTGTTGTTGGACTTGCAAATGATCTTGAACGGTATCAACAACTACAAGAACCCA

AAGTTGACCAGAATGTTGACCTTCAAGTTCTACATGCCAAAGAAGGCTACCGAATTGAAG

CACCTGCAGTGTCTAGAGGAACAGTTGAAGCCATTGGAAGAAGTCCTGAACTTGGCTCAA

TCTAAGAACTTCCACTTGAGACCAAGAGACTTGATCTCTAACATCAACGTTATCGTTTTG

GAATTGAAGGGTTCTGAAACCACCTTCATGTGTGAATACGCTGACGAAACCGCTACCATC

GTTGAATTCTTGAACAGATGGATCACCTTCTGTCAATCTATCATCTCTACCTTGACC

3. A method according to Claim 2, wherein said secretory leader and processing signal is derived from α-factor.

4. A method according to Claim 3, wherein said α-factor secretory leader and processing signal

ar modified at least by removal of the glu-ala dipeptide codons.

5. A method according to Claim 3, wherein said DNA sequence is synthesized by producing two or more dsDNA fragments with flanking regions for linking to other sequences, which fragments are joined together to produce the entire sequence.

6. A DNA construct comprising in the direction of transcription yeast recognized transcriptional regulatory signals and a secretory leader sequence and processing signal; in reading frame with said secretory leader and processing signals, a synthetic DNA sequence encoding for the human IL2 gene having at least a plurality of codons preferentially utilized by yeast; stop codon(s); and, a transcriptional terminator.

7. A DNA construct according to Claim 6, wherein said DNA sequence is as follows:

GCTCCAACCTCTTCCTCTACCAAGAAGACCCAGCTGCAATTG

GAACACTTGTTGTTGGACTTGCAATGATCTTGAACGGTATCAACAACTACAAGAACCCA

AAGTTGACCAGAATGTTGACCTTCAAGTTCTACATGCCAAAGAAGGCTACCGAATTGAAG

CACCTGCAGTGTCTAGAGGAAGAGTTGAAGCCATTGCAAGAAGTCCTGAACTTGGCTCAA

TCTAAGAACTTCCACTTGAGACCAAGAGACTTGATCTCTAACATCAACGTTATCGTTTTG

GAATTGAAGGGTTCTGAAACCACCTTCATGTGTGAATACGCTGACGAAACCGCTACCATC

GTTGAATTCTTGAACAGATGGATCACCTTCTGTCAATCTATCATCTCTACCTTGACC

8. A DNA construct according to Claim 7, wherein said secretory leader and processing signal is derived from the α-factor secretory leader and processing signal.

9. A DNA construct according to Claim 8, wherein said α-factor secretory leader and processing signal are modified at least by removal of the glu-ala dipeptide codons.

FIG._1.

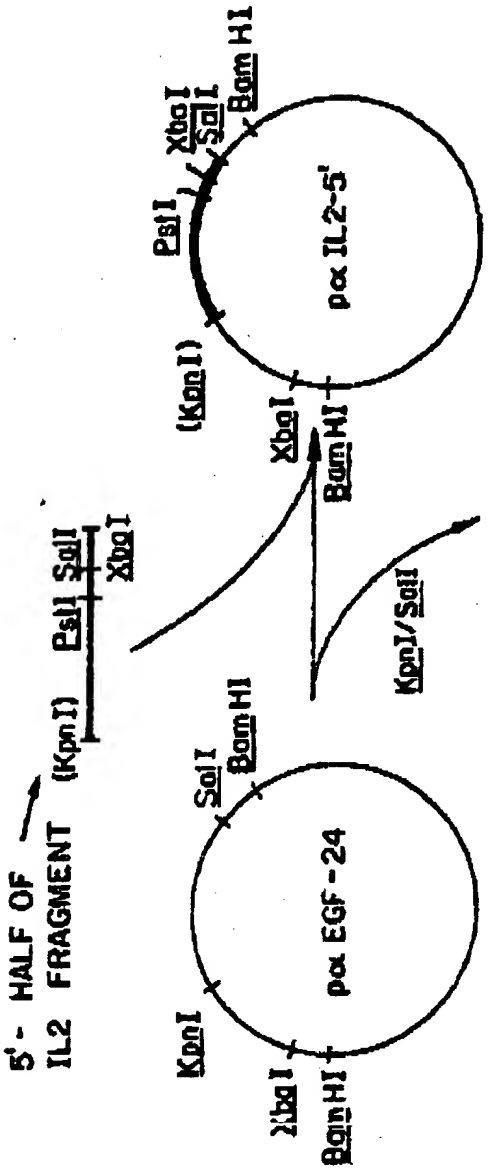

FIG._2.

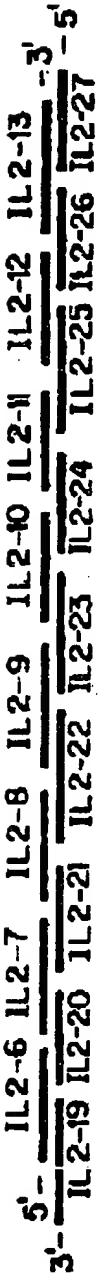

FIG._3.

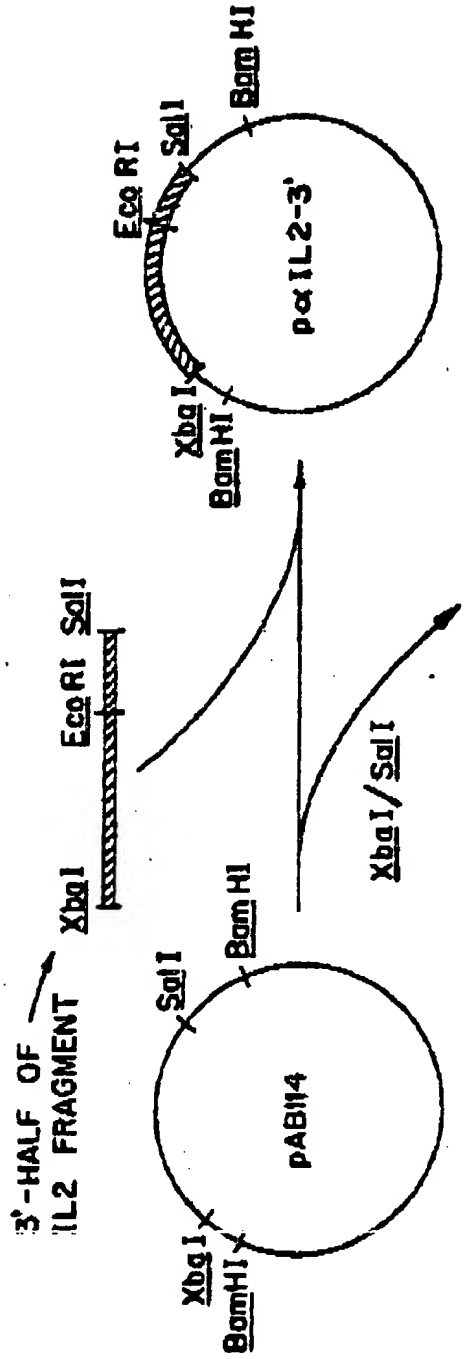

FIG._4.

SUBSTITUTE SHEET

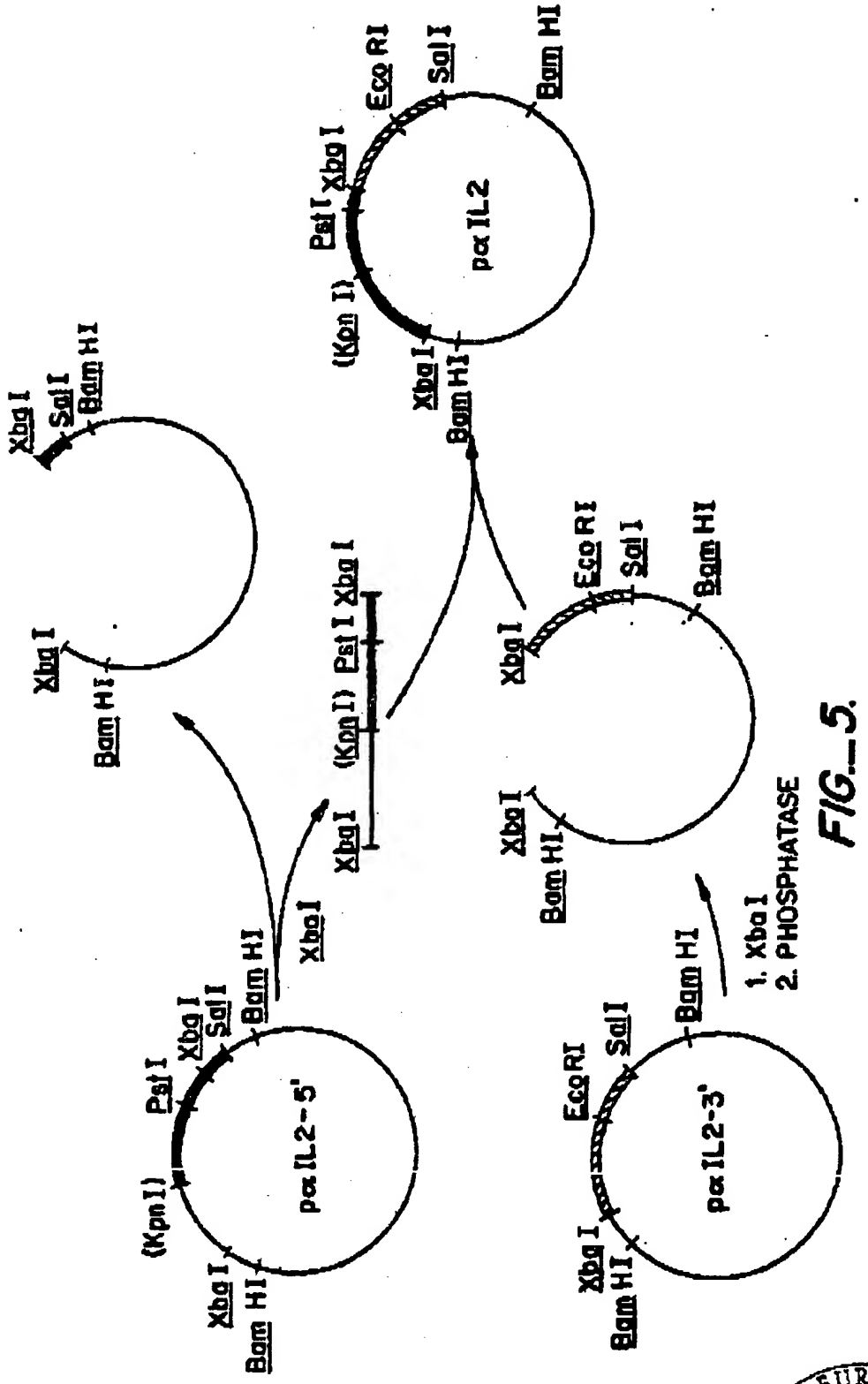

FIG_5.

SUBSTITUTE SHEET